

INVESTIGATION OF THE METHYLATION STATUS OF *FGF2* GENE IN
PERIODONTAL TISSUES AS DETERMINANTS OF HEALING RESPONSE AFTER
IMPLANT SURGERY

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ABSTRACT

Travis Joe Whitley: INVESTIGATION OF THE METHYLATION STATUS OF *FGF2* GENE IN PERIODONTAL TISSUES AS DETERMINANTS OF HEALING RESPONSE AFTER IMPLANT SURGERY
(Under the direction of Silvana Barros)

Periodontal wound healing is a complex process involving a series of interactions of growth factors, including FGF-2. Previous studies have suggested that the *FGF2* gene may be down-regulated in diseased periodontal tissues. Aberrant gene expression in smokers and diabetics has also been reported. The aims of this study were to determine the association of *FGF2* methylation level with wound healing after implant surgery and the association of smoking and diabetes with *FGF2* methylation. Thirty-four included subjects were distributed into control, smoking and diabetic groups. During the implant surgery, gingival tissue biopsies were collected for real-time PCR analysis. This study demonstrated that FGF-2 hyper-methylation and associated gene down-regulation were associated with smoking status and diabetes.

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INTRODUCTION AND BACKGROUND

Osseointegration and Dental Implant Wound Healing

Wound healing involves a series of complex events that are coordinated and sequential. Many cells, signaling pathways, and growth factors play integral roles in this process with precise coordination. In implant bone healing, these processes lead to osseointegration; the direct structural and function connection between ordered, living bone and the surface of a load-carrying implant (Brånemark 1983). Animal and human studies have demonstrated the timeline of events that occur following surgical placement of a dental implant (Berglundh 2003; Lang 2011). Four phases are seen in the implant healing process that correlates with similar soft tissue healing events: hemostasis, inflammation, proliferation, and remodeling. Briefly, 2 hours after implant installing, red blood cells and inflammatory cells dominate the space between implant threads. The implant is stabilized by contact between the adjacent osteotomy bony walls and threads of the implant. After 4 days, fibroblasts and new blood vessels filled the area with osteoclastic activity seen on the old bone. One week showed the presence of new bone formation on the implant surface. Two weeks marked the presence of woven bone formation with bridging between the parent bone and implant surface occurring at 4 weeks. At six weeks, the remodeling phase had begun with the formation of primary and secondary osteons (Lang 2011). During both the inflammatory and proliferative phases, release of growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF or FGF-2), and transforming growth factor are critical elements in

the recruitment of cells, formation of new extracellular matrix, and angiogenesis to the implant surface.

Fibroblast Growth Factor -2 (FGF-2) and Wound Healing

Fibroblast growth factor (FGF) is protein involved with many aspects of wound healing. FGF was isolated completely in 1974 from bovine pituitary glands (Gospodarowicz 1974). FGF exists in two different isometric points, 1 and 2. While one was known as the acidic FGF (aFGF) or FGF1, the other became known as the basic FGF (bFGF) or FGF-2 (Bohlen et al. 1984). The entire nucleotide sequence of human FGF-2 was isolated two years later (Abraham et al. 1986).

As a member of the heparin-binding growth factor family, FGF-2 is found in various body tissues and exerts a range of biological effects on cells from different embryological origins. It acts as a potent angiogenic molecule *in vivo* and *in vitro*, aiding in wound healing and facilitating events necessary for re-vascularization. During this process, it causes proliferation of macrophages and other cell types involved in repair (Bikfalvi et al. 1997, Lee et al. 2010).

FGF-2 has also been shown to play an important role in development, repair and regeneration of periodontal tissues (Lee et al. 2010, Murakami 2011). FGF-2 is secreted by macrophages and endothelial cells during the process of wound healing. During the proliferative healing phase, FGF-2 stimulates migration and proliferation of endothelial cells. In addition to producing collagen, FGF-2 differentiates myofibroblasts to induce wound contraction. Deposition of extra-cellular matrix components and production of collagen are also key roles played by FGF-2. While promoting angiogenesis, FGF-2 exhibits potent mitogenic ability on mesenchymal cells within the periodontal ligament

(Murakami et al. 1999). During the bone remodeling phase, it can stimulate migration of mesenchymal progenitor cells which ultimately differentiate into osteoblasts and cementoblasts (Murakami et al. 1999).

Several animal (Murakami et al. 1999, Murakami et al. 2003, Takayama et al. 2001) and human (Kitamura et al. 2008, Kitamura et al. 2011, Kitamura et al. 2015) studies have shown promising results in the use of FGF-2 for periodontal regeneration. In 1999, Murakami surgically created 2- and 3-wall intrabony defects along with Class II furcation defects in beagle and primate models. Six to eight weeks following surgical access and topical application of recombinant FGF-2, significantly greater cementum, periodontal ligament and bone formation was seen in the FGF-2 sites compared to control sites. Histological evidence of regeneration was also portrayed in this study. Epithelial down growth, ankylosis or root resorption were not observed in FGF-treated sites (Murakami et al. 1999). In 2001, another animal study was conducted by Takayama on non-human primates. Surgically-created Class II furcation defects were treated with either 0.1% human recombinant FGF-2, 0.4% rhFGF-2, carrier alone or no treatment. Their results demonstrated a significant increase in periodontal regeneration clinically and histologically with the higher dose (0.4%) of rhFGF-2 compared to other treatment groups (Takayama et al. 2001). In 2003, Murakami surgically created Class II furcation defects in beagle dogs and treated with rhFGF-2 compared with control sites. Histomorphometrically, greater amounts of new cementum and bone formation, along with creation of PDL fibers was found compared to the control sites (Murakami et al. 2003).

In 2008, a Phase II randomized, controlled, double-blind clinical trial was conducted by Kitamura at 13 study facilities with 74 subjects. These subjects presented with 2- or 3-wall vertical bone defects and randomly assigned to four treatment groups: carrier alone, carrier + 0.03% FGF-2, carrier + 0.1% FGF-2, or carrier + 0.3% FGF-2. The results demonstrated a significantly greater rate of increase in alveolar bone height between the group with 0.3% FGF-2 compared to the carrier alone group at 36 weeks. No differences were noted for clinical attachment level or alveolar bone gains between groups (Kitamura et al. 2008). Three years later, the group performed a subsequent larger clinical trial which involved 253 patients. In this study, 200 μ L of 0%, 0.2%, 0.3% or 0.4% FGF-2 was administered to 2- or 3-walled bony defects at the time of a modified Widman flap surgery. All FGF-2 sites demonstrated significant higher percentage of bone fill at 36 weeks (Kitamura et al. 2011).

In 2015, Kitamura again published results on two randomized, placebo-controlled clinical trials comparing rhbFGF-2 and enamel matrix derivative (EMD) in periodontal regeneration for infrabony defects. The first study included 323 subjects who randomly received 0.3% FGF-2 or placebo during flap surgery. At 36 weeks, a significantly greater percentage of bone fill was seen for the FGF-2 group vs placebo (37% vs 21.5%). The second study included 264 subjects randomly assigned to receive either 0.3% FGF-2, EMD, or flap alone for treatment of infrabony defects. At 36 weeks, linear alveolar bone growth for the FGF-2 and EMD groups were 1.93 and 1.36 mm, respectively, which was statistically significantly different.

While FGF-2 has been shown to significantly increase regeneration of periodontal tissues *in vitro* and *in vivo*, scarce data exists on its role in subjects with diabetes.

Recently, Bizenjima published an animal study on the healing of surgical periodontal defects in diabetic rats treated with FGF-2. The group created defects on maxillary first molars, which were treated with either a control carrier or FGF-2. Histological and radiographic analyses at 2 and 4 weeks showed a significant increase in bone volume and trabecular patterns for the FGF-2 group vs the control group (Bizenjima, 2015).

Role of FGF-2 and Bone Healing

While FGF-2 is well known for its role in angiogenesis, several studies have also identified a role for it in osteogenic pathways (Marie, 2003). The growth and function of mesenchymal stem cells from bone marrow seems to be stimulated by FGF-2 (Murikami 2011). FGF-2 plays a key role in not only cell recruitment and proliferation in the area of injury, but differentiation of osteoblastic cell lineages to induce bone formation. FGF-2 null mice have been shown to demonstrate low bone mass and decreased bone formation as they age (Montero 2000, Behr 2010). Evidence reveals that FGF-2 has a significant impact in bone formation and remodeling by upregulation of the RANK – RANKL pathway (Fei, 2013). FGF-2 has also been shown to have positive effects on bone fracture healing. Animal and human studies have demonstrated that with increasing doses of FGF-2 applied to long bone fractures, an increase in osteocytes, bone mineral density, and bone repair is seen (Kawaguchi 1994, Furuya 2014, Tanaka, 2012). Mutations in FGF receptors have been associated with a number of craniofacial disorders, including achondroplasia and craniosynostosis. These processes are important events in the healing after dental implant placement and can greatly impact osseointegration. Recently, a beagle dog model was used to study the effect of FGF-2 application on implant osseointegration. Histomorphometry was used to examine the new bone area and bone-

to-implant contact (BIC) at 4, 8, and 12 weeks. The study demonstrated that FGF-2 application around dental implants significantly increased the bone area and BIC at 4 weeks compared to control implants. Implant stability quotient (ISQ) measurements were also obtained with significantly higher values for the FGF-2 group at 4, 8, and 12 weeks compared to controls (Nagayasu-Tanaka 2016).

DNA Methylation – An Overview

The term “epigenetics” was first used by Conrad H. Waddington, a developmental biologist and evolutionist, in the middle of the twentieth century to describe heritable changes in patterns of gene expression without changes in the DNA sequence (Barros & Offenbacher 2009). He stressed that phenotype was a product of an interaction between the organism’s genetic sequence and its environment. Epigenetics refers to events which chemically modify certain DNA regions, leading to alteration of the chromatin and ultimately silencing or activation of a gene (Gomez et al. 2009). Various mechanisms of epigenetic alterations were discovered in the past half century including DNA methylation, post-translational histone modifications, or chromatin structural changes. Among them, DNA methylation is the most extensively studied mechanism in mammals (Barros & Offenbacher 2009).

DNA methylation occurs when a methyl group binds to a cytosine residue within the cytosine-guanine dinucleotide (CpG) regions (Barros & Offenbacher 2009, Gomez et al. 2009, Barros & Offenbacher 2014). Methylated CpG regions interfere with the access of transcription factors to the promoter region, thereby silencing the gene (Barros & Offenbacher 2009 and 2014, Wilson 2008) (Figure 1). This DNA methylation phenomenon has important regulatory functions in controlling normal cellular processes.

During corneal wound healing, hyper-methylation of the maspin gene promoter causes down-regulation of maspin synthesis in corneal stromal cells, enabling these cells to undergo phenotypic changes to fibroblasts and myofibroblasts (Horswill et al. 2008). In the periodontal ligament of aged individuals, the decreased expression of collagen $\alpha 1(1)$ gene is associated with elevated levels of methylation at most CpG sites in the proximal and distal regions of the promoter (Ohi et al. 2006).

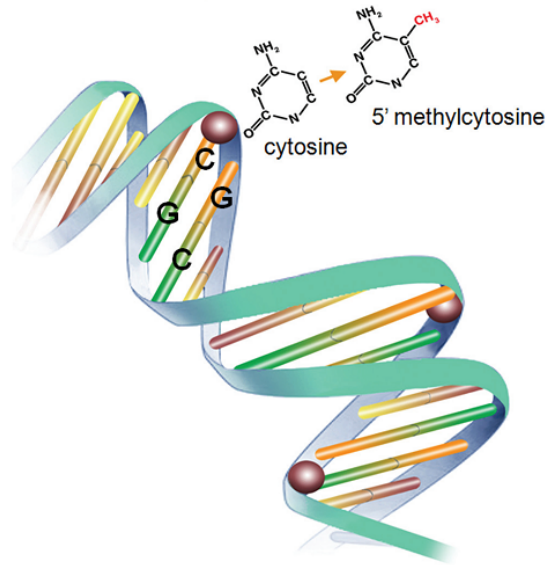


Figure 1. The DNA Methylation Process (adapted from Barros & Offenbacher 2009 and reproduced with permission).

In addition to physiological responses, DNA methylation has also been demonstrated to play a role in pathological cellular processes. Alteration in the methylation status at the promoter region of tumor suppressor genes was demonstrated to have an association with cancer (Wilson 2008). Persistent periodontal inflammation can also cause DNA methylation, which inactivates suppressors of cytokine signaling, resulting in exaggerated cytokine production. This lack of suppression renders an individual susceptible the development of a proinflammatory state, increasing the risk for

periodontitis (Gomez et al. 2009). In our laboratory, we discovered that chronic periodontal disease is associated with increased DNA methylation of the prostaglandin-endoperoxide synthase-2 (*PTGS2* or *COX2*) promoter, resulting in dampening of *COX2* expression. The methylation level of the *PTGS2* promoter from diseased gingival biopsies demonstrated a 5.06-fold increase when compared with non-diseased samples ($p = 0.03$) (Zhang et al. 2010).

Although DNA methylation has been identified as important components in various physiological and pathological processes, its role in periodontal wound healing has not yet been investigated.

Smoking and Its Effect on the Periodontium

According to the National Health Interview Survey performed by the U.S. Census Bureau in 2014, 17% of the US adult population was current smokers (CDC 2015). The detrimental effects of smoking on systemic health are widely known. Smoking is a major risk factor for heart attacks, strokes, chronic obstructive pulmonary diseases, emphysema and cancer.

The adverse effects exerted by smoking on periodontal health have been demonstrated by compelling evidence. Locally, smoking causes a reduction in the gingival blood flow, thereby reducing the number of circulating leukocytes and the amount of oxygen reaching the gingiva (Giannopoulou et al. 1999). Systemically, smoking reduces serum immunoglobulin G production (Johnson & Guthmiller 2007) and inhibits chemotactic and phagocytic activities of peripheral blood neutrophils (Giannopoulou et al. 1999, Palmer et al. 2005). These actions locally reduce the inflammatory host responses.

Nicotine, a cytotoxic and vasoactive substance present in cigarette smoke, contributes to most of the negative effects caused by smoking. In addition to those already mentioned, it has been shown to inhibit the proliferation and attachment of PDL fibroblasts, as well as alkaline phosphatase production in cultures (Giannopoulou et al. 1999). Reduced collagen production and increased collagenase activity in gingival fibroblast cultures exposed to nicotine has been demonstrated (Tipton & Dabbous 1995). The production of superoxide anion and hydrogen peroxide by neutrophils is inhibited by smoking. Oxygen uptake by neutrophils and release of superoxide and IL-1 β by monocytes in vitro is decreased in smokers (Palmer et al. 2005, Pabst et al. 1995). Smoking also stimulated osteoclastic differentiation in a porcine marrow cell model (Henemyre et al. 2003), impaired angiogenesis in rats (Pinto et al. 2002), inhibited expression of various growth factors in rabbits (Theiss et al. 2000) and elevated production of inflammatory cytokines in humans (Tymkiw et al. 2011).

Table 1. Proposed Mechanisms for the Negative Periodontal Effects of Smoking (adapted from Johnson & Guthmiller 2007 and reproduced with permission)

Proposed Mechanisms for the Negative Periodontal Effects of Smoking
- Decreased immunoglobulin G2 production
- Chronic reduction in blood flow and vascularity
- Increased prevalence of potential periodontal pathogens
- Shift in neutrophil function towards destructive activities
- Negative effects on cytokine and growth factor production
- Inhibition of fibroblast growth, attachment and collagen production

Smoking is a well-established risk factor for periodontitis. Its relationship with periodontitis was examined in the Third National Health and Nutrition Examination Survey (NHANES III) (Tomar & Asma 2000). In this large-scale epidemiological study, 12,329 U.S. adults were included. Results found that current smokers were 4 times as likely as never smokers to have periodontitis, and 42% of periodontitis cases (6.4 million cases) were attributable to current smoking. Smokers have also been shown to have reduced alveolar bone height, increased interproximal bone loss, increased attachment loss, higher number of missing teeth, increased probing depths and fewer sites with bleeding on probing compared to non-smokers (Bergstrom et al. 1991, Haffajee & Socransky 2001, Bergstrom 2004, Machuca et al. 2000, Natto et al. 2005, Rosa et al. 2008).

Smoking also has a negative impact on outcomes of periodontal therapy. A meta-analysis evaluated the effect of smoking on non-surgical therapy and found that probing depth reduction was significantly reduced in smokers compared with non-smokers (Labriola et al. 2005). A review of various forms of periodontal surgical therapies indicated that smokers exhibited only 50-75% as much improvement in probing depth and attachment level as non-smokers (Johnson & Guthmiller 2007). These sub-optimal therapeutic outcomes are likely attributable to the compromised healing response seen in smokers.

Smoking can increase the risk of failure or complications in implant therapy. Based on a review article, smokers were found to have at least twice the implant failure rate compared to non-smokers. The survival rates were summarized to be 80-100% and 93-98% for smokers and non-smokers respectively (Johnson & Guthmiller 2007). However, majority of these studies were retrospective in nature. In 2007, a meta-analysis evaluating the effect of smoking on implant failure showed a significantly enhanced risk for implant failure among smokers with an overall odds ratio of 2.25 (Strietzel, 2007). Another review in 2009 showed a 3.6 to 4.6 increased risk of peri-implantitis in smokers compared to non-smokers (Heitz-Mayfield, 2009).

The benefits of smoking cessation have been demonstrated for non-surgical periodontal therapy (Chambrone, 2013), periodontal health (Fiorini, 2014) and risk of implant failure (Bain, 1993, 1996). In 2013, Chambrone et al, published a systemic review and meta-analysis on the effects of smoking cessation on the outcomes of non-surgical periodontal therapy. In the limited number of studies included, an improvement in clinical attachment levels following treatment could be seen. The authors concluded

that a benefit to smoking cessation was seen and outcomes following treatment in patients who stopped smoking were improved. In a systematic review, Fiorini et al, found that in patients who quit smoking, less progression of clinical attachment loss, a decrease in radiographic bone loss and periodontal probing depths could be seen during the course of periodontal treatment and maintenance (Fiorini, 2014).

Some studies have demonstrated a relationship between smoking cessation and dental implant failures. A significantly greater number of implant failures were seen in smokers than in non-smokers (11.3% vs 4.8%) in a study of over 2,000 Brånemark implants over a 6-year period (Bain, 1993). A follow-up study of a cessation protocol where patients quit smoking 1 week prior and 8 weeks following dental implant placement was also completed by the same author. Bain demonstrated a significantly lower failure rate for those who followed the protocol compared to those who continued to smoke (11.8% vs 38.5%) (Bain, 1996). It is important to note that the implants used in the two previous studies were machined surface implants. Several studies have demonstrated no significant difference between implant failures in smokers vs non-smokers when roughened surface implants are placed (Kumar, 2002; Balshe, 2008; Chambrone, 2014); however, cigarette smoke and its components negatively impact wound healing, which can affect outcomes of periodontal and dental implant therapy and cessation should be discussed and implemented when possible.

DNA Methylation and Smoking

The majority of studies on this topic focused on the impact of smoking on gene methylation in bronchial or lung tissues in lung cancer patients (Belinsky et al. 2002, Soria et al. 2002, Divine et al. 2005, Liu et al. 2007, Han et al. 2009, Tekpli et al. 2011).

Most of these studies demonstrated a positive correlation between cigarette smoking and abnormal promoter methylation patterns. Recently, Zhang performed a meta-analysis on the association between smoking and *p16^{INK4a}* methylation in surgically resected tumor tissues from non-small cell lung carcinoma (NSCLC) patients. Nineteen cross-sectional studies were evaluated. The group found an odd ratio of 2.25 (CI = 1.81 – 2.80) of *p16^{INK4a}* methylation in NSCLC patients with smoking habits compared to those who did not smoke. This positive association was even stronger in an Asian population (Zhang et al. 2011). In a separate study, Murphy investigated the relationship between *in utero* exposure to cigarette smoking, DNA methylation and birth weight of infants in 418 pregnant women. Results showed that approximately 20% of smoking-related low birth weight in male infants was mediated by DNA methylation at the insulin-like growth factor-2 differentially methylated region (Murphy et al. 2011).

To date, there are only two studies that have investigated the impact of smoking on the DNA methylation status of the periodontal tissues. Both of them were conducted on patients with chronic periodontitis. The first study analyzed the methylation status in the *IL-8* gene promoter in oral mucosal cells from 41 healthy non-smokers, 30 smokers with chronic periodontitis and 40 non-smokers with chronic periodontitis (Oliveira et al. 2009). The second study analyzed the methylation status in the promoter region of the toll-like receptor *TLR2* and *TLR4* genes in gingival tissues from 11 healthy non-smokers, 11 smokers with chronic periodontitis and 12 non-smokers with chronic periodontitis (De Oliveira et al. 2011). In both studies, smokers were defined as a subject who consumed at least 5 cigarettes per day for a minimum of 5 years. Unlike the previous studies on lung cancer, however, neither of them succeeded in demonstrating a correlation between

smoking and methylation status of the gene of interest. The inconsistent results may be attributed to the lower smoking doses of these subjects. More investigations regarding the contribution of smoking to epigenetic modification in periodontal tissues are needed.

Diabetes and Its Effect on the Periodontium

Diabetes is a common metabolic disease that affects over 29 million Americans and 415 million adults worldwide (ADA, 2014). Because of disturbances in insulin production and/or insulin resistance of cells and tissues, abnormal metabolism of sugar, fat and protein are seen in diabetics (Southerland 2006). The condition is associated with an increased incidence of hypertension, obesity, stroke, heart failure, blindness, limb amputations, end-stage renal disease, birth complications and sexual dysfunction (Winer & Sowers 2004). In 2015, the American Diabetes Association reported 12% of global health expenditure (\$673 billion) is spent on diabetes. It was also projected that by 2040, 1 in 10 people (642 million) will have diabetes (American Diabetic Association 2014).

The adverse effects exerted by diabetes on the periodontium have been supported by consistent evidence. As early as 1967, Glickman noted delayed healing response of alloxan-induced diabetic rats after removing the marginal gingiva of their left maxillary and mandibular incisors (Glickman 1967). Devlin noted that ten days after molar extraction in diabetic rats, the formation of collagen in extraction sockets is inhibited, resulting in delayed wound healing and increased alveolar destruction (Devlin 1996). Nishimura cultured human periodontal ligament cells in mediums of different glucose levels. The group noted cells cultured in glucose-containing mediums underwent changes in morphology and experienced suppressed chemotactic response to platelet-derived growth factor (Nishimura 1996). Desta created surgical wounds on the palatal gingiva of

type 1 and type 2 diabetic mice. The group noted the diabetic mice had less epithelial wound coverage, less new connective tissue formation and reduced fibroblast density five days after the surgery (Desta 2010). These studies demonstrated the ability of diabetes to compromise periodontal wound healing.

Diabetes is a well-known risk factor for periodontal disease. In 1990, Nelson investigated in the rate of periodontal disease in 2273 Pima Indians with non-insulin dependent diabetes mellitus (NIDDM) and found it to be 2.6 times higher than those without (Nelson 1990). In 1991, Emrich also assessed the relationship between diabetes mellitus and oral health status in 1342 Pima Indians. Their results indicated that Type 2 diabetics had an odds ratio of 2.81 for attachment loss and an odds ratio of 3.43 for bone loss when compared to non-diabetic subjects (Emrich 1991). In 1998, Taylor studied data obtained on the oral health status of 362 Gila River Indians and found the NIDDM subjects were at 4.23 times higher risk of alveolar bone loss (Taylor 1998).

Taylor later performed a comprehensive review of relevant literature from 1960 to 2001 on diabetes and periodontal diseases. Among 21 reports on patients with type 1 diabetes, 8 reports on type 2 diabetes, 10 studies on both types of diabetes and 8 studies in which the type of diabetes were not specified, all except one demonstrated greater prevalence, extent, or severity of at least one clinical parameter of the periodontium in diabetic subjects. In addition, based on four of the five studies included, the review suggested poor glycemic control to be a significant risk factor for poor periodontal health (Taylor 2001). In 2006, Khader conducted a meta-analysis on 18 cross-sectional studies and 3 prospective cohort studies. The group demonstrated that the diabetic subjects had more gingival inflammation and periodontal disease as measured by the mean values of

gingival index, probing depth and clinical attachment loss (Khader 2006). The relationship between diabetes mellitus and destructive periodontal disease was further strengthened when Chavarry performed a systematic review in 2009. Among the 49 cross-sectional studies included in the review, 27 detected more periodontal diseases in diabetic subjects compared with non-diabetic subjects. A significant association was detected in clinical attachment level and periodontal pocket depth for type 2 diabetic subjects. The group concluded that type 2 diabetes should be considered as a risk factor of periodontitis (Chávarry 2009). Recently, Morita showed that the risk of developing periodontal disease was associated with HbA1c level. As the level of HbA1c increased, the risk of periodontitis also increased. This again confirmed the effects diabetes can exert on the periodontium (Morita 2012).

Various biological mechanisms have been proposed to explain the impact diabetes has on the periodontium. Sustained hyperglycemia has also been demonstrated to cause proteins to become glycated and form advanced glycation end products (AGEs). AGEs often form on collagen, and this increases collagen cross-linking and formation of collagen macro-molecules. These macro-molecules are resistant to enzyme degradation (Monnier 1996). Elevated levels of MMPs have been found in the periodontium of diabetic patients. These MMPs can degrade the newly formed collagen (Sorsa 1992). The net effect of decreased collagen synthesis, increased MMP production and resultant AGE formation is the rapid degradation of newly formed collagen and the accumulation of collagen macro-molecules (Mealey 2006). These changes affect the normal homeostatic collagen turnover and alter the wound healing responses in the periodontium.

Abnormal growth and impaired regeneration of vessels have been demonstrated in the periodontium of diabetic subjects. AGEs have been shown to accumulate on the walls of blood vessels (Wautier 1998). This ultimately impairs tissue perfusion and wound healing. When AGEs form on collagen in bone, altered osteoblastic differentiation (McCarthy 2001) and diminished bone healing can be observed (Santana2003).

While most culture studies demonstrated bacterial compositions in periodontally diseased sites in both diabetic and non-diabetic patients were similar, alterations in immune and inflammatory responses of the host were believed to be the major cause of increased extent and severity of periodontal diseases in diabetic patients (Mealey 2006). Neutrophil adherence, chemotaxis and phagocytosis have been shown to be defective, resulting in decreased bacterial killing and increased periodontal destruction (Manouchehr-Pour 1981, McMullen 1981). On the other hand, monocytes and macrophages are often hyper-responsive, resulting in significant increase in production of pro-inflammatory cytokines and mediators (Naguib2004). Hyperglycemia has been observed to inhibit osteoblast proliferation and collagen production, which lead to less bone formation and formation of weakened bone (Lu 2003, Lu 2003, Gooch 2000). Finally, AGEs have been demonstrated to activate a receptor called “receptor of AGEs” (RAGE) on various cells (Schmidt 1996, Schmidt 1999). The AGE-RAGE interaction on monocytes can increase cellular oxidant stress and activate a transcription factor called nuclear factor kappa B (Schmidt 1996). This results in increased production of pro-inflammatory cytokines, ultimately causing increased tissue destruction in the periodontium.

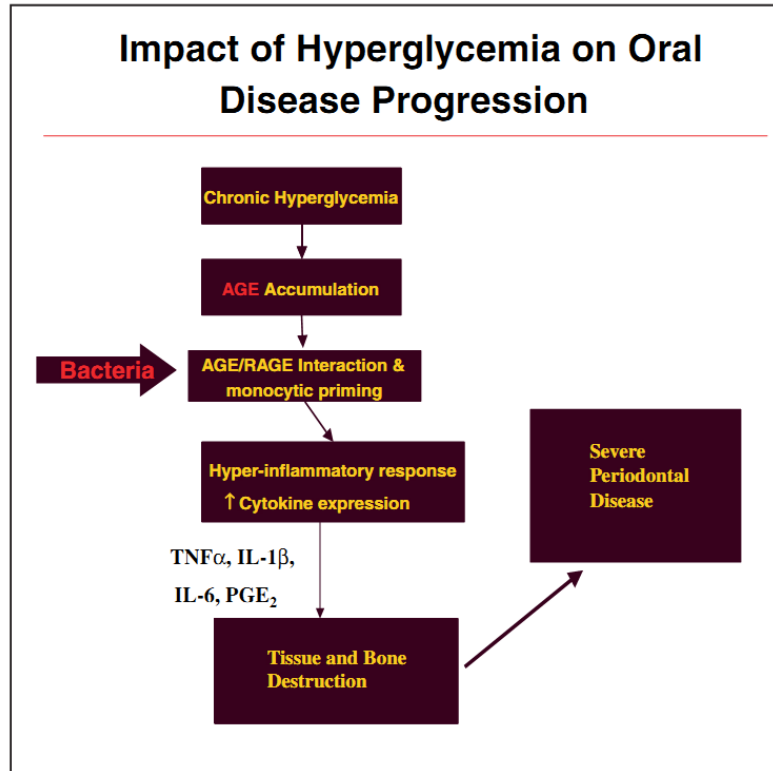


Figure 2. Possible Linkage between Diabetes and Periodontal Disease Severity (adapted from Southerland et al. 2006 and reproduced with permission).

Similar to smoking, diabetes can increase the risk of failure of implant therapy. de Morais performed an animal study in which dental implants were placed in the tibia of 40 adult rats. The group stated that diabetes impaired bone density around the implants (de Morais 2009). Javed and Romanos performed a systematic review of 18 studies selected between 1982 and 2009. They reported that implant osseointegration was negatively affected by poorly controlled diabetes but occurred successfully in patients with optimal glycemic control (Javed 2009).

DNA Methylation and Type II Diabetes

Currently, no investigations have been initiated on the impact of diabetes on the DNA methylation status of the periodontal tissues. Only limited studies that have examined the relationship among epigenetic status in target tissues, insulin production and development of type 2 diabetes exist. One study reported that DNA methylation in the promoter of transcriptional co-activator, *PRARGCIA*, was elevated in pancreatic islets from patients with type 2 diabetes compared with healthy subjects. *PRARGCIA* expression was shown to correlate inversely with the degree of DNA methylation, and thus was reduced in pancreatic islets of diabetic patients (Ling et al. 2008). Another study examined the role of DNA methylation in the regulation of mouse and human insulin gene expression. The CpG sites in both mouse *Ins2* and human *INS* promoters were found to be hypomethylated in healthy pancreatic beta cells. When these CpG regions were methylated, insulin promoter activity was suppressed by almost 90% (Kuroda et al. 2009). Review articles on epigenetics in diabetic kidney disease also highlight the implications of DNA methylation in development of diabetes, fibroblast proliferation and fibrosis in injured kidneys, and vascular complications of diabetic patients (Ling & Groop 2009, Reddy & Natarajan 2011). Recently in 2014, Lou et. al, demonstrated that the expression of proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α in diabetic rat aortas was significantly higher compared to control rats (Lou, 2014). Many studies have demonstrated a hyperinflammatory state in diabetics that contributes to decreased wound healing and other complications.

Insulin resistance, a condition often seen in type 2 diabetes, may also be related to DNA methylation. Zhao recently performed a twin study that examined the association

between global DNA methylation of Alu repeats in peripheral blood leukocytes and insulin resistance in 84 pairs of monozygotic twins. Results showed that methylation levels of all four CpG sites were individually associated with insulin resistance ($p = 0.026$). Genetic factors did not confound this association. This study again confirmed the possible role of DNA methylation in pathogenesis of type 2 diabetes (Zhao et al. 2011).

AIMS

The overall goal of this study is to identify alterations of methylation status of the *FGF2* gene and how this methylation affects its expression after implant surgeries in healthy individuals, smokers and diabetics.

The specific aims include:

1. To determine the methylation status of the *FGF2* gene and gene expression during wound healing after implant placement.
2. To determine whether smoking and diabetes are associated with differential methylation of *FGF2* gene.

HYPOTHESES

It is hypothesized that the methylation status of the *FGF2* gene can affect the level of FGF-2 secreted during wound healing phase after dental implant surgery. It is also hypothesized there exists a difference in methylation levels of *FGF2* gene in healthy subjects, smokers and diabetics that can interfere with wound healing.

MATERIALS AND METHODS

Study Population

This study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. Subjects of age 18 – 70 years were recruited into this case-control study (Appendix I. Supplementary Materials and Methods). The subjects were placed into one of three groups according to their medical condition. Group 1 (control) consisted of non-smokers and non-diabetics. Group 2 (smoking category) consisted of smokers who were not diabetic. Group 3 (diabetic category) consisted of diabetics who were not smokers. The smoking group could be current smokers or former smokers who had quit smoking within the last 10 years. The target number of recruitment was 20 for the control group, 12 for the smoking group and 12 for the diabetic group. All subjects received at least one dental implant placement within the study time frame. Implants were placed as one-stage, either on edentulous ridges or in extraction sockets. Recruited subjects must have probing depth ≤ 4 mm or 5mm without bleeding on probing for all teeth in the same quadrant of implant placement. Individuals were excluded from the study if they had chronic disease with oral manifestations (e.g. Crohn's disease, lupus erythematosus, Behcet's disease, etc), exhibited gross oral pathology, have used antibiotics or NSAIDs within 2 weeks of the screening examination, have undergone chronic treatment (i.e. two weeks or more) with any medication known to affect periodontal status (e.g. phenytoin, calcium, antagonists, cyclosporine, warfarin) within 1 month of screening examination, had systemic conditions except smoking and diabetes

that are known to affect the periodontal status, had active infectious diseases such as hepatitis A – E, HIV or tuberculosis, and were known to be pregnant or breastfeeding

Gingival Tissue Biopsies, Gingival Crevicular Fluid (GCF) Collection and Implant Stability Quotient (ISQ) Measurements

For each enrolled subject, a screening periodontal examination was performed within two weeks of the implant surgery or on the day of implant placement. Plaque index (PI), gingival index (GI), probing depth (PD), percentage of bleeding on probing (BOP %) and clinical attachment level (CAL) were recorded for all teeth in the same quadrant of implant placement. Glycosylated hemoglobin (HbA1c) levels of the diabetic study subjects were also measured by a DCA 2000[®]+ Analyzer (Bayer Inc., Toronto, ON, Canada). On the day of surgery and prior to implant placement, 8 GCF samples were collected from the mesiolingual, mesiobuccal, distolingual and distobuccal surfaces of the two teeth that are in the closest proximity to the implant site. GCF samples were collected using filter paper strips (Pro Flow, Inc., Amityville, NY). During the implant surgery of each subject, a gingival biopsy was obtained over the implant site using a 4mm punch biopsy blade or a scalpel blade and divided in half. One half of the sample was to be used for DNA methylation analysis, the other for mRNA gene expression analysis. Biopsy samples for mRNA expression were immediately placed in RNeasy[®] (Applied Biosystems / Ambion Inc., Austin, TX) overnight at 4°C and stored at -80°C 24 hours later. Samples for DNA methylation were immediately stored at -80°C. One sample for mRNA expression from the diabetic group was lost during lab processing.

Study subjects returned for follow-up visits at 2 weeks, 4 weeks and 6 weeks after implant surgeries. GCF samples were collected from the same sites, wound healing indices

(WHI) as indicators of degree of soft tissue healing were recorded for the implant sites, and GI and PI were scored for all teeth in the same quadrant of implant placement during these three visits. Degree of osseointegration in terms of implant stability was measured using Osstell ISQ instrument (Osstell Inc., Linthicum, MD) at 4 weeks and 6 weeks after implant surgeries. WHI measurements, ISQ data, and GCF samples were collected but due to the purpose of this paper, the findings will not be presented. This data will be used for future analyses.

RNA Isolation and Quantitative Real-Time Reverse Transcription PCR

RNA was isolated from the collected gingival tissue samples using a TissueLyser LT (Qiagen, Valencia, CA) and a RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA was quantified using the NanoDrop (Thermo Scientific, Wilmington, DE). For each sample, a volume of 300 ng of RNA was used to generate complementary DNA (cDNA) through reverse transcription reactions using the SuperScript[®] VILO[™] cDNA Synthesis Kit (Invitrogen[™], Life Technologies[™], Grand Island, NY). Quantitative real-time PCR (qRT-PCR) was performed with 2µL of synthesized cDNA, 7.5µL of Taqman[®] Universal PCR Master Mix, .75µL of Taqman[®] Gene Expression Assay Mix for *FGF2* gene and 5.5µL of ddH₂O in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Carlsbad, CA). mRNA expression level was normalized using GAPDH as a housekeeping gene.

DNA Isolation, Selective Digestion and Quantitative Real-Time PCR

Genomic DNA was isolated from the collected gingival tissue samples using DNeasy Mini Kit (Qiagen, Valencia, CA). The DNA was quantified using the NanoDrop (Thermo Scientific, Wilmington, DE). 250 ng of genomic DNA isolated from gingival tissue samples was digested using EpiTect Methyl II DNA Restriction Kit (Qiagen Cat#

335452). DNA was aliquoted into four equal portions. Each portion was subjected to a different reaction: Mock digest (no digestion), methylation sensitive digest (digestion of unmethylated and partially methylated DNA only), methylation dependent digest (digestion of methylated DNA only), and double digest (digestion of both methylated and unmethylated DNA). DNA digestion was allowed to take place at 37°C for 6 hours to overnight. Following the digestion inactivate the enzymes at 65 °C for 20 minutes. Digested DNA was then quantified by qPCR using EpiTect Methyl II PCR Primer Assay for Human FGF-2 (CpG Island ID: 111236, Cat #: EPHS111236-1A). qRT-PCR was performed using 12.5µL of RT SYBR Green qPCR Master Mix, 1µL of PCR primer mix for *FGF2* gene (Catalogue no. MePH06727-1A, SABiosciences, Frederick, MD), 1µL of each digested DNA and 10.5µL of ddH₂O in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Carlsbad, CA). Data analysis was done using integrated Excel-based templates provided by the manufacturer, which provided gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. Unmethylated (UM) represents the fraction of input genomic DNA no methylated CpG sites in the amplified region of a gene. Methylated (M) represents the fraction of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene. A relative methylation fold change to controls was reported.

Statistical Analysis

Chi-square test was used to compare gender and race. Fisher's Exact Test was used to compare implant sites. ANOVA test was used to compare age and BMI among the three study groups. The ANOVA test was also applied for comparison in mRNA expression and DNA methylation among various groups. Linear regression was used to

determine the association of DNA methylation with mRNA expression. The threshold for statistical significant difference was set at a p-value ≤ 0.05 . All analyses were performed with SAS (SAS Institute, Cary, NC).

RESULTS

Participant Recruitment Process

From September 2010 to January 2016, a total of 236 patients attending the UNC Graduate Periodontal Clinic for implant placement were screened. 202 patients were not recruited into the study based on a variety of reasons (Table 2). A total of 34 participants were successfully recruited.

Table 2. Participant Recruitment Process

Recruitment Process	Number (%) of Subjects
Screening	236(100.0%)
Reason for Disqualification:	202 (85.5%)
Ex-smoker	73 (30.9%)
Age > Upper Limit	36 (15.3%)
Unable to Collect Tissue Sample	24 (10.2%)
Not interested	15 (6.4%)
Unable to Obtain Consent	9 (3.8%)
< 2 teeth Remained in Same Quadrant	5 (2.1%)
Use of NSAIDs < 1 month	4 (1.7%)
Smoking < 5 pack-years	3 (1.3%)
Others	33 (14.0%)
Recruited	34 (14.4%)

Study Population Demographics

The 34 recruited participants included 20 control subjects, 7 smokers and 7 diabetics. The mean participant age varied from 47.9 to 58.9 years with a standard deviation ranged from 1.8 to 2.9 (Table 3). Majority of the participants were Caucasians, and more female participants were recruited in the control and smoking groups. BMI was recorded for all participants with the mean values for groups ranging from 28.3 to 33.9 kg/m². Statistical significance was not found with gender and race among the three study groups; however, the mean age ($p=0.003$) and BMI ($p=0.03$) for the diabetic group were statistically significantly higher than the control group (Table 3).

One subject did not successfully complete the study. The subject who withdrew belonged to the control group. She had an implant failure at 2 weeks after the surgery, and the cause of failure was not contributable by the study. The implant site was grafted and implant placement will be reattempted in the near future. The tissue sample collected from this subject was used in the DNA and RNA analyses. Tissue samples from three subjects were obtained and used in the DNA and RNA analyses, but the follow-up visits were not completed. Two of these subjects were smokers with the other being diabetic.

Table 3. Study Participant Demographics (Total n = 34)

Groups			
Variables	Control (n=20) N (%)	Smoking (n=7) N (%)	Diabetic (n=7) N (%)
Number	20 (58.8%)	7 (20.6%)	7 (20.6%)
Mean age (StdDev)	47.4 (1.8)	54.1 (2.9)	58.3 (2.9)*
Gender:			
Male	8 (40%)	1 (14%)	4 (57%)
Female	12 (60%)	4 (86%)	3 (43%)
Race:			
Caucasian	14 (70%)	7 (100%)	5 (71%)
African American	4 (20%)	0	2 (29%)
Asian	1 (5%)	0	0
Other	1 (5%)	0	0
BMI (StdDev)	28.2 (1.3)	27.1 (2.0)	33.9 (2.0)*

***p < 0.05:** significantly different from control group

Selected Implant Sites

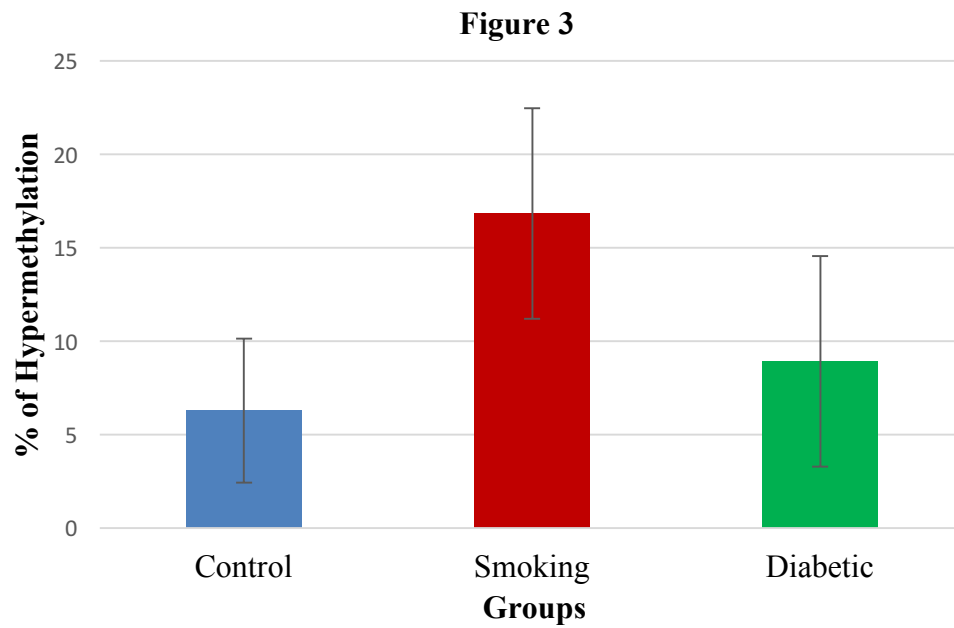
The selected implant sites for participants in all 3 groups were listed on Table 4. Majority of implants for the control group was placed in the molar and premolar regions, especially in the mandibular molar area. All implants for the diabetic group were placed in the maxillary arch, with the majority being in the anterior sextant. No statistical significance was found with respect to implant site among the three study groups ($p=0.09$).

Table 4. Implant Sites of Study Participants (Total n = 34)

Groups				
Implant sites	Control (n=20) N (%)	Smoking (n=7) N (%)	Diabetic (n=7) N (%)	P-Value
Maxillary molar	3 (15%)	1 (14.5%)	2 (28.5%)	0.09
Maxillary premolar	5 (25%)	2 (28.5%)	1 (14.5%)	
Maxillary anterior	1 (5%)	1 (14.5%)	4 (57%)	
Mandibular molar	9 (45%)	2 (28.5%)	0	
Mandibular premolar	2 (10%)	1 (14.5%)	0	
Mandibular anterior	0	0	0	

Differential DNA Methylation of FGF2 Gene in Smokers and Diabetic Participants

A comparison among the control subjects, smokers and diabetic participants indicated increased methylation of *FGF2* gene was present in the smoking and diabetic groups (Figure 3). The percentages of hyper-methylation in the gingival tissues of the control, diabetic and smoking groups were 6.29 ± 3.85 , 8.92 ± 5.64 and 16.84 ± 5.64 respectively. The p-values were 0.70 and 0.34 for the gingival samples from diabetics and smokers respectively when compared to the control samples. This indicated the difference in the methylation level of *FGF2* gene between smoking and control groups was not statistically significant; however, a trend towards higher methylation of *FGF2* gene was seen for smokers and diabetics compared to controls.



***p-value for smoking group= 0.34**

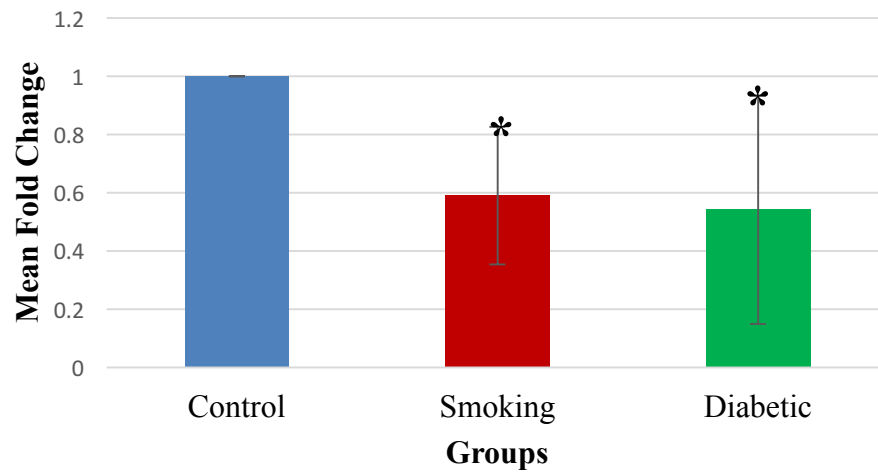
***p-value for diabetic group= 0.70**

Figure 3. Methylation Level of *FGF2* Gene in Smokers or Diabetics in Comparison to Control Subjects.

Differential mRNA Expression of FGF2 Gene in Smokers and Diabetic Participants

A comparison among the control subjects, smokers and diabetic participants indicated there was a down-regulation pattern of mRNA expression of *FGF2* gene in the smoking and diabetic groups (Figure 4). The fold changes for the control, smoking and diabetic groups were 1.0 ± 0 , 0.59 ± 0.23 and 0.54 ± 0.39 respectively. In other words, the gingival tissues in the smoking and diabetic groups had 0.6 and 0.5-fold decrease in *FGF2* gene expression respectively compared to gingiva from the control group. The p-values of the corresponding comparisons for the diabetic and smoking groups were 0.03 and 0.04 respectively, indicating the differences were statistically significant. This data shows a statistically significant decrease in gene expression for *FGF-2* in the smoking and diabetic groups compared to control subjects.

Figure 4



***p-value < 0.05**

Figure 4. Messenger Level of *FGF2* Gene in Smokers or Diabetics in Comparison to Control Subjects

DISCUSSION

In this study, hyper-methylation of *FGF2* gene was consistently observed in the gingiva from smoking and diabetic subjects relative to the gingiva from healthy subjects. The level of mRNA expression of the *FGF2* gene is down-regulated in gingiva from smokers and diabetics. Overall, differential methylation patterns of the *FGF2* gene among gingiva from healthy individuals, smokers and diabetics were associated with levels of *FGF2* gene expression.

Limited literature relating smoking and diabetes with DNA methylation of other genes could be identified. Regarding smoking, majority of studies focused on bronchial or lung tissues in lung cancer patients (Belinsky et al. 2002, Soria et al. 2002, Divine et al. 2005, Liu et al. 2007, Han et al. 2009, Tekpli et al. 2011). Most studies demonstrated a positive correlation between cigarette smoking and abnormal methylation patterns. In a meta-analysis performed by Zhang on the association between smoking and *p16^{INK4a}* methylation in tumor cells from lung carcinoma patients, more than a 2-fold increase of *p16^{INK4a}* methylation in NSCLC patients with smoking habits was noted. The group later found the association to be stronger in an Asian population (Zhang et al. 2011). Lin demonstrated nicotine-derived nitrosamine ketone, a cigarette smoke carcinogen, was capable of influencing the expression of DNA methyltransferase 1 (DNMT1) and ultimately leading to hypermethylation of *p16^{INK4a}* in a dose-dependent manner in human

lung tumor tissues (Lin et al. 2010). However, in two studies performed by Oliveira (Oliveira et al. 2009) De Oliveira et al. 2011) regarding the impact of smoking on the DNA methylation status of *IL-8*, *TLR2* and *TLR4* gene promoters in the periodontal tissues, both failed to demonstrate a correlation between smoking and methylation of these gene promoters. Smoking dose likely had a significant role in the inconsistent results. In the lung cancer study, smokers were defined as subjects who demonstrated at least a 5 pack-year history, but 1.25 pack-years was the threshold in Oliveira's studies.

Regarding diabetes, literature has also been identified. One study reported that DNA methylation in the promoter of transcriptional co-activator, *PRARGC1A*, was elevated in pancreatic islets from patients with type 2 diabetes (Ling et al. 2008). Another study demonstrated methylation of the CpG sites in both mouse *Ins2* and human *INS* promoters in pancreatic beta cells suppressed the insulin promoter activity by as much as 90% (Kuroda et al. 2009). A third study demonstrated the methylation levels of CpG sites in the Alu repeats of peripheral blood leukocytes were associated with insulin resistance (Zhao et al. 2011). Recently, Singh et. al completed a case control study analyzing diabetic foot ulcers from Type 2 diabetic subjects. Biopsies were taken from the wound margins and used for real time-PCR analysis of *TLR2* and the methylation status of the *TLR2* promoter. *TLR2* mRNA expression was reduced in Type 2 diabetic foot ulcers compared to controls and tended to increase with the severity of the wounds. While the methylation status of the *TLR2* gene was not significantly different for the different grades of wounds, the CpG sites investigated were totally or partially methylated in majority of the diabetic foot ulcer cases. This study concluded that *TLR2* down regulation in diabetic subjects may lead to chronic wound development and plays a significant role

in wound healing (Singh, 2015). In general, existing evidence supports findings from our current study, that smoking and diabetes are associated with hyper-methylation pattern of *FGF2* gene.

A statistically significant association was not detected for DNA methylation of *FGF2* gene in smokers and diabetics compared to control subjects; however, the smoking group demonstrated almost two times greater % methylation than the diabetic group. This can partly be explained by the severity of the systemic condition of subjects included. Subjects in the smoking group were mostly heavy smokers, with two being former smokers who had quit within the last 10 years. While the dose-response relationship exerted by smoking on periodontal health has been well documented in literature (Bergstrom et al. 1991, Machuca et al. 2000, Haffajee & Socransky 2001, Bergstrom 2004, , Natto et al. 2005, Rosa et al. 2008), the degree of DNA hyper-methylation in the gingival tissues can be expected to be higher than subjects who have never smoked. The mean DNA methylation and gene expression could have been affected, but in a more significant way due to smokers having increased smoking duration, amount, and current smoking status. On the other hand, the diabetic subjects had HbA1C levels of 7.0% or less, indicating that their diabetic condition was controlled. Diabetic patients who are controlled have been shown to exhibit healthier periodontal status than those with poor glycemic control (Taylor 2001). It is reasonable to observe the increased methylation pattern of *FGF2* gene in the gingiva of smokers, even more so than diabetics. Although the methylation results did not reach statistical significance, a pattern was observed.

In the current study, the methylation of *FGF2* gene in gingival samples was detected with the EpiTect Methyl DNA Methylation PCR system (Methyl-Profiler™,

SABiosciences Corporation, Frederick, MD). Unlike the bisulfate-based methods, this method detects remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme. These enzymes can cleave a minimum of three unmethylated CpG sites and three methylated CpG sites respectively in the promoter region. Following digestion, the remaining DNA fragments of *FGF2* gene are quantified by real-time PCR using primers that bind to the promoter region of interest. The exact number of CpG sites cleaved by each enzyme, the CpG locations on the *FGF2* gene and the sequence of the primers are proprietary to the manufactory company, and thus they could not be obtained for result evaluation.

FGF-2 can up-regulate many genes and affect various downstream reactions, but the exact mechanisms by which the *FGF2* gene is regulated remain unclear. No studies at the present have investigated how smoking can affect RNA expression of *FGF2* gene during wound healing. Only one study could be identified to support the inhibitory effect that smoking has on the activity of FGF-2 protein. Pauwels and his group (Pauwels et al. 2010) evaluated whether cigarette smoke triggered pulmonary and systemic pentraxin 3 (PTX3) expression in vivo in a murine model. PTX3, a soluble pattern recognition receptor, is produced in response to pro-inflammatory cytokines IL-1 β and TNF- α and mediates angiogenesis by influencing FGF-2 activity. They detected elevated levels of PTX3 and reduced mRNA expression of *FGF2* at 4 weeks and 24 weeks after exposure to cigarette smoke. These findings could be related to binding of FGF-2 protein by the N-terminal domain of PTX3, which inhibited the angiogenic activity of FGF-2.

Potential biological mechanisms of how diabetes down-regulates *FGF2* RNA expression have been proposed. Werner and his group (Werner et al. 1994) demonstrated

RNA expression of *FGF2* was reduced in diabetic mice compared with normal mice during the period of wound re-epithelialization and granulation tissue formation. They stated this observation could be resulted from delayed infiltration of acute inflammatory cells into the wound area, impaired ability for diabetic macrophages to synthesize FGF-2, and reduced level of serum components in diabetic mice to induce *FGF2* expression. Lu and his group (Lu et al. 2008) found a circulating electronegative low density lipoprotein (LDL) named L5 in type 2 diabetes. L5 induced endothelial cell apoptosis by suppressing *FGF2* expression. *FGF2* expression exerted most of its effects by activating the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. Once activated by *FGF2*, the phosphorylated Akt activated endothelial nitric oxide synthase, which is essential for survival of endothelial cell. Lu suggested FGF-2 was auto-regulated by Akt activation through a positive feedback loop. They isolated L5 from type 2 diabetic patients, and found L5 caused reduction in Akt phosphorylation causing a decrease in the transcription of the *FGF2* gene. He concluded that L5 could potentially impair FGF-2 dependent re-endothelialization and could play a role in diabetes-associated wound impairment and atherosclerosis.

FGF-2 plays an important role in bone formation, differentiation and metabolism (Marie 2003, Kawaguchi et al. 2000). It is produced by osteoprogenitor cells and mature osteoblasts and accumulates in bone extra-cellular matrix. Being a potent mitogen for mesenchymal cells, it directs these cells to commit to the osteoblastic lineage (Kawaguchi et al. 2000). The regulatory effects of FGF-2 on genes controlling osteoblast differentiation were recognized with main contribution from studies on genetically modified mice and human mutations. The binding of FGFs with osteoblasts induces

signaling pathways involving the expression of transcription factors such as Runx2 and AP-1 that activate downstream osteoblast differentiation genes (Marie 2003). Coffin (Coffin et al. 1995) discovered that over-expressing FGF-2 in mice induced abnormal long bone formation, and Montero (Montero et al. 2000) later noted bone formation was inhibited by deactivating FGF-2.

Using *Fgf2*-null mice, Fei and his group (Fei et al. 2011) demonstrated a role of FGF2 in osteoblastogenesis through Wnt signaling. Wnts are a family of 19 glycoproteins secreted in mammals, and were well demonstrated to positively regulate osteoblast differentiation and bone formation (Krishnan et al. 2006). The group found that mRNA and protein expression for Wnt10b and β -catenin decreased significantly in *Fgf2*-null mice, and the addition of exogenous FGF-2 promoted β -catenin accumulation in the nucleus of bone marrow stromal cells while also increasing their mineralization.

The effects of FGF-2 can be enhanced with bone morphogenetic protein-2 (BMP-2). Rat marrow-derived mesenchymal stem cells exposed with FGF-2 and BMP-2 induced expression of osteocalcin mRNA and formation of bone-like nodules earlier than either factor alone (Hanada et al. 1997). Supplementing mesenchymal stromal cell culture sequentially with FGF-2 before BMP-2 resulted in high ALP activity, elevated bone-specific osteocalcin expression and abundant bone matrix formation (Maegawa et al. 2007). Adult rabbits underwent intertransverse process fusions at L4-L5 and implanted with mesenchymal stem cells. Only the group with both BMP-2 and FGF-2 addition demonstrated bone in-growth at 6 weeks, and this group had a higher 6-week fusion rate than the groups with only BMP-2 or FGF-2 (Minamide et al. 2007). Recently, FGF-2 has been demonstrated to reduce expression of the BMP receptor 1A on murine

osteoprecursor cells, influencing osteoblast differentiation (Park 2011). In addition to BMP-2, a review written by Marie in 2003 also provided evidence to support interaction of FGF-2 with other biological factors, such as cAMP and TGF- β to enhance osteogenesis (Marie 2003).

The ability of FGF-2 to regulate bone remodeling is supported by studies that demonstrated bone resorption caused by FGF-2 stimulation in cell cultures (Kawaguchi et al. 2000, Simmons & Raisz 1991, Kawaguchi et al. 1995, Chikazu et al. 2001). In these studies, FGF-2 was shown to modulate osteoclast differentiation through various mechanisms. The indirect mechanism involved FGF-2 activating cyclo-oxygenase 2 (COX2) and producing prostaglandin, leading to the induction of the receptor activator of nuclear factor κ B ligand (RANK-L) in osteoblasts (Kawaguchi et al. 2000, Kawaguchi et al. 1995). This effect was abolished by NSAIDs (Kawaguchi et al. 2000). On the other hand, the direct mechanisms involved FGF-2 binding to FGF receptor type 1, a tyrosine kinase receptor on osteoclasts and their precursors, to induce bone resorption and prevent differentiation (Kawaguchi et al. 2000, Chikazu et al. 2001) (Chikazu et al. 2001). FGF-2 has been shown to play a key role in affecting molecular interactions and intracellular mechanisms in osteoblasts to induce osteoblastic differentiation and enhance osteogenesis.

Several limitations can be identified in the study. 1) The principle one is the small sample sizes in the smoking and diabetic groups. The target number of recruitment was 20 for the control group, 12 for the smoking group and 12 for the diabetic group; however, the control group was the only category that met the goal. 2) Another important one is the cross-sectional design used to evaluate impact of smoking and diabetes on

DNA methylation. 3) Smoking history and dosage were self-reported, and HbA1c was not evaluated in the other two non-diabetic groups. This led to reporting biases and potential for ex-smokers, current smokers and undiagnosed diabetics to be recruited into the wrong group. 4) Subjects in the smoking and diabetic groups were not stratified according to their smoking doses or HbA1c levels. Evaluation of dose-response relationships was not possible. This dosage could have impacted the amount of DNA methylation seen in each sample. 5) Bone structure and implant size nor surface were not controlled. Although all implant surfaces were roughened, a difference in surface modification could play a role in recruitment of growth factors, migrations of cells, and the rate of implant healing and osseointegration. Future investigations should 1) increase the sample sizes, 2) increase number of follow-up visits to assess wound healing more closely, 3) address causality and dose response of smoking and control of diabetes with DNA methylation, 4) specify the bone quality of the patients, implant type and implant size to be included in the study, and 5) evaluate DNA methylation, mRNA expression and protein expression of other biological factors such as platelet-derived growth factor, transforming growth factor- β or interleukin-1 β .

In conclusion, association patterns of differential methylation of *FGF2* gene in smokers and diabetics were identified in this study. Hyper-methylation levels of *FGF2* gene coincided with down-regulation of its mRNA expression. Systemic and environmental factors were shown to have epigenetic effects on the expression of the *FGF2* gene which could affect their healing response. A potential role of the epigenetic modification of genomic DNA in the biological process of periodontal wound healing was found based on the results of this study. The evaluation of DNA methylation patterns

of known biomarkers may allow clinicians to more predictably assess the risk and complications of dental implant treatment. Further studies are needed to determine the effect of smoking and diabetes and their impact on DNA methylation, gene expression, and healing of the periodontium.

APPENDIX I

SUPPLEMENTARY MATERIALS AND METHODS

Institutional Review Board, University of North Carolina at Chapel Hill

IRB Study Number 10-1184

1. Clinical Study Design

This is a longitudinal case-control study of 6-8 weeks in duration. Clinical parameters and biological samples were collected from healthy subjects, smokers and diabetics during the course of implant therapy. The study was performed in a single masked or blinded manner. All laboratory assessments were performed without knowledge of the subjects' type of treatment and health condition.

2. Human Subjects

Subjects were recruited from the Graduate Periodontology clinic in the School of Dentistry of University of North Carolina. All subjects had at least one implant placement in their treatment plan that was set up in the Graduate Periodontology clinic. They were placed into one of three groups according to their smoking and diabetic statuses. Group 1 (control) consisted of subjects who have never smoked and who were never diagnosed with diabetes. Group 2 (smoking category) consisted of subjects who were currently smoking and had at least 5 pack-year of smoking history, but were never diagnosed with diabetes. Group 3 (diabetic category) consisted of subjects who have never smoked, but were currently being treated with medications and/or insulin for type II diabetes. All subjects had healthy periodontium. Smoking history and diabetic status were self-

reported. The level of control of the diabetic condition was confirmed by HbA1c test within 3 months prior to screening. Screening and follow-up examinations were performed in the General Oral Health Center in the School of Dentistry. Implant surgery was performed in the Graduate Periodontology clinic.

The inclusion criteria of study participation included: (1) males or females between the age of 18 and 70 years (inclusive), (2) must be able and willing to follow study procedures and instructions, (3) must have read, understood and signed an informed consent form, (4) must be in good general health, (5) must have one or more implant placements as their future treatment needs, (6) must fit into one of the study groups as listed previously, and (7) must have probing depth ≤ 4 mm or 5mm without bleeding on probing for all teeth at the same quadrant of implant placement as determined by two calibrated examiners .

Individuals were not enrolled into the study if they presented with the following exclusion criteria: (1) chronic disease with oral manifestations (e.g. Crohn's disease, lupus erythematosus, Behcet's disease), (2) gross oral pathology, (3) use of either antibiotics or NSAIDs within 2 weeks prior to screening examination, (4) chronic treatment (i.e. two weeks or more) with any medication known to affect periodontal status (e.g. phenytoin, calcium, antagonists, cyclosporin, warfarin) within 1 month prior to screening examination, (5) systemic conditions, except smoking and diabetes, that were known to affect the periodontal status, (6) active infectious diseases such as hepatitis, HIV or tuberculosis, and (7) known to be pregnant or breastfeeding.

Recruited subjects must not present major changes in medical status during the entire study period for continuation of the protocol. There must have no infection at the

implant site for the entire duration of the study. Subjects not meeting this criterion would be withdrawn. Infection around an implant would be reported as an adverse event and subject would be followed until it was resolved.

3. *Clinical Study Method*

The following procedures were performed on study participants during a total of 5 visits within a study period of 6-8 weeks.

Visit 1 (≤ 2 weeks before implant surgery):

- Study personnel obtained a signed written informed consent from the subject.
- Study personnel obtained medical history and demographics related to the patient.
- Study personnel performed HbA1C test on all subjects in the diabetic group (if a test result within past 3 months was not available). A drop of blood was obtained by finger prick and was loaded into a DCA 2000 HbA1C reagent cartridge, which was then processed immediately by a DCA 2000®+ Analyzer (Bayer Inc., Toronto, ON, Canada) for HbA1C level.
- Patient vital signs (blood pressure and pulse), height and weight were recorded.
- A dental examiner performed a general oral examination for presence of any abnormalities.
- A dental examiner scored all teeth in the same quadrant of implant placement for plaque index, gingival index, probing depth, percent bleeding on probing and clinical attachment level.

Visit 2 (Implant surgery):

- Study personnel updated medical history and obtained vital signs.

- A dental examiner performed a general oral examination for presence of any abnormalities.
- The dental examiner collected 8 GCF samples from the mesiolingual, mesiobuccal, distolingual and distobuccal surfaces of the two teeth that were in the closest proximity to the implant site. Samples were collected using filter paper strips (Pro Flow, Inc., Amityville, NY).
- The dental examiner collected 1 biopsy at the site of implant placement for quantitative PCR, DNA methylation sequence analysis and differential methylation analysis. The biopsy sample was obtained with a 4mm punch biopsy blade or a scalpel blade. It was removed in the same way as how discarded tissues will be removed. It was removed at the beginning of the surgery as the clinician exposed the underlying bone at the implant site, or before suturing when the clinician reduced redundant tissues to achieve better flap closure.

Visits 3-5 (2 weeks \pm 2 days, 4 weeks \pm 2 days, 6 weeks \pm 2 days after surgery):

- Study personnel updated medical history and obtained vital signs.
- A dental examiner performed a general oral examination for presence of any abnormalities.
- A dental examiner scored all teeth in the same quadrant of implant placement for plaque index and gingival index.
- The dental examiner recorded wound healing indices (WHI) as indicators of degree of soft tissue healing for the implant site.
- The dental examiner tested degree of osseointegration in terms of degree of implant stability using Osstell ISQ instrument. (Visit 4 and 5 only)

- The dental examiner collected 8 GCF samples in the same areas and using the same method as indicated previously.

Table 5. Schedule of Procedures by Visit

Week	Visit 1 -2 to -1	Visit 2 0	Visit 3 2	Visit 4 4	Visit 5 6
Procedure:					
Informed Consent	X				
Medical/Dental History	X	X	X	X	X
Demographics	X				
Vital Signs	X	X	X	X	X
HbA1C Test (for diabetic group only)	X				
Oral Examination	X	X	X	X	X
PI, GI	X		X	X	X
PD, BOP, CAL	X				
WHI			X	X	X
Implant Stability Measurement				X	X
Biopsy Sample		X			
GCF Sample		X	X	X	X
Saliva Sample		X	X	X	X
Adverse Experience	X	X	X	X	X

4. *Clinical Periodontal Assessments*

Clinical examiners were calibrated prior to commencement of the study. Clinical parameters assessed in the periodontal examination included plaque index, gingival index, probing depth, bleeding on probing, and clinical attachment level. Clinical parameters were measured using a manual University of North Carolina (UNC-15) periodontal probe. These parameters were measured at six periodontal sites per tooth (i.e., mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) and at all teeth in the same quadrant where implant placement is desired (including the third molar).

Silness and Løe Plaque Index (PI) (SILNESS & LOE 1964):

PI was defined as the relative amount of supragingival plaque and was recorded on an ordinal scale of 0-3.

- 0- No plaque in the gingival area.
- 1- A film of plaque adhering to the free gingival margin and the adjacent tooth. The plaque may be recognized only by running a probe across the tooth surface
- 2- Moderate accumulation of soft deposits within the gingival pocket and on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye.
- 3- Abundance of soft matter within the gingival pocket and/or on the gingival margin and the adjacent tooth surface.

Løe and Silness Gingival Index (GI) (LOE & SILNESS 1963):

GI was defined as the degree of gingival inflammation and was recorded on an ordinal scale of 0-3.

- 0- Normal gingiva.
- 1- Mild inflammation (slight change in color, slight edema); no bleeding on palpation (i.e., sulcular sweep).
- 2- Moderate inflammation (redness, edema, glazing); bleeding on palpation (i.e., sulcular sweep).
- 4- Severe inflammation (i.e., marked redness, edema); ulceration, tendency to spontaneous bleeding.

Probing depth (PD):

PD was defined as the linear distance from the gingival margin (GM) to base of the pocket. If a PD reading fell between two millimeter readings, the rule was to round down and the lower of the two readings was recorded.

Bleeding on Probing (BOP):

BOP was defined as the presence or absence of bleeding to manual probing and was recorded as a dichotomous variable.

- 0- No bleeding within 10 seconds after probing.
- 1- Bleeding within 10 seconds after probing.

Clinical attachment level (CAL):

CAL was defined as the linear distance from the cemento-enamel junction (CEJ) to base of the pocket. If a CAL reading fell between two millimeter readings, the rule was to round down and the lower of the two readings was recorded.

Wound healing indices (WHI):

WHI was defined as the degree of soft tissue healing and consisted of four indices. Each index was recorded on an ordinal scale of 0-3.

Degree of Edema

- 0- No gingival edema.
- 1- Mild gingival edema.
- 2- Moderate gingival edema.
- 3- Severe gingival edema.

Degree of Erythema

- 0- No erythema.
- 1- Mild erythema.
- 2- Moderate erythema.
- 3- Severe erythema.

Degree of Pain on Palpation

- 0- No pain.
- 1- Mild pain.
- 2- Moderate pain.
- 3- Severe pain.

Degree of Flap Closure

- 0- No fibrin line or folding line in the incision area.
- 1- Fine fibrin line or folding line in the incision area.
- 2- Fibrin clot in the incision area.
- 3- Partial or complete necrosis of flap.

Implant Stability:

Implant stability at 4 and 6 weeks after implant placement was measured with a device called Osstell ISQ instrument. This instrument used resonance frequency analysis

as a method to measure implant stability. It consisted of a wireless SmartPeg, a hand-held probe and the Osstell meter. The SmartPeg was first attached to the implant. The hand-held probe then stimulated it magnetically. The displayed Implant Stability Quotient (ISQ) reflected the degree of stability. The scale ranged from 1 to 100. The higher the ISQ, the more stable the implant. The overall average value of all implants over time was approximately 70 ISQ.

REFERENCES

- (U.S.) C. f. D. C. (2009) State-specific smoking-attributable mortality and years of potential life lost--United States, 2000-2004. *MMWR.Morbidity and mortality weekly report* 58, 29-33.
- Abraham J. A., Whang J. L., Tumolo A., Mergia A., Friedman J., Gospodarowicz D. & Fiddes J. C. (1986) Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *The EMBO journal* 5, 2523-2528.
- American Diabetes Association (2014). National Diabetes Statistics Report. June 10 2014. Diabetes.org
- Association A. (2008) Economic costs of diabetes in the U.S. In 2007. *Diabetes care* 31, 596-615.
- Aykol G., Baser U., Maden I., Kazak Z., Onan U., Tanrikulu-Kucuk S., Ademoglu E., Issever H. & Yalcin F. (2011) The Effect of Low-Level Laser Therapy as an Adjunct to Non-Surgical Periodontal Treatment. *Journal of periodontology* 82, 481.
- Bain C. A., Moy P. K. (1993) The Association Between the Failure of Dental Implants and Cigarette Smoking. *Int J Oral Maxillofac Implants* 1993; 8: 609-615.
- Bain C. A. (1996) Smoking and Implant Failure - Benefits of a Smoking Cessation Protocol. *Int J Oral Maxillofac Implants* 1996; 11: 756-759.
- Balshe A. A., Eckert S. E. (2008). The Effects of Smoking on the Survival of Smooth- and Rough-Surface Dental Implants. *Int J Oral Maxillofac Implants* 2008; 23: 1117-1122.
- Barros S. P. & Offenbacher S. (2009) Epigenetics: connecting environment and genotype to phenotype and disease. *Journal of dental research* 88, 400-408. doi: 10.1177/0022034509335868
- Barros S. P. & Offenbacher S. (2014) Modifiable risk factors in periodontal disease: Epigenetic regulation of gene expression in the inflammatory response. *Periodontology* 2000, Vol. 64, 2014, 95-110.
- Behr B, Panetta NJ, Longaker MT, Quarto N. Different endogenous threshold levels of Fibroblast Growth Factor-ligands determine the healing potential of frontal and parietal bones. *Bone* 2010; 47(2): 281-94.
- Belinsky S. A., Palmisano W. A., Gilliland F. D., Crooks L. A., Divine K. K., Winters S. A., Grimes M. J., Harms H. J., Tellez C. S., Smith T. M., Moots P. P., Lechner J. F., Stidley C. A. & Crowell R. E. (2002) Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. *Cancer research* 62, 2370-237

Berglundh T, Abrahamsson I, Lang NP, Lindhe J. De Novo alveolar bone formation adjacent to endosseous implants. *Clin Oral Implants Res* 2003; 14: 251-262.

Bergstrom J. (2004) Influence of tobacco smoking on periodontal bone height. Long-term observations and a hypothesis. *Journal of clinical periodontology* 31, 260-266. doi: 10.1111/j.1600-051X.2004.00475.x

Bergstrom J., Eliasson S. & Preber H. (1991) Cigarette smoking and periodontal bone loss. *Journal of periodontology* 62, 242-246.

Bikfalvi A., Klein S., Pintucci G. & Rifkin D. B. (1997) Biological roles of fibroblast growth factor-2. *Endocrine reviews* 18, 26-45.

Bizenjima T., Seshima F., Ishizuka Y., Takeuchi T, Kinumatsu T., Saito A. Fibroblast growth factor-2 promotes healing of surgically created periodontal defects in rats with early, streptozotocin-induced diabetes via increasing cell proliferation and regulating angiogenesis. *J Clin Periodontol* 2015; 42: 62-71.

Bohlen P., Baird A., Esch F., Ling N. & Gospodarowicz D. (1984) Isolation and partial molecular characterization of pituitary fibroblast growth factor. *Proceedings of the National Academy of Sciences of the United States of America* 81, 5364-5368.

Brånemark PI. Osseointegration and its experimental studies. *J Prosthet Dent* 1983; 50: 399-410.

Center for Disease Control and Prevention (2015). Summary Health Statistics Tables for US Adults: National Health Interview Survey, 2014.

Chambrone L., Preshaw P.M., Rosa E.F., Heasman P.A., Romito G.A., Pannuti C. M., Tu Y. K. (2013) Effects of Smoking Cessation on the Outcomes of Non-Surgical Periodontal Therapy: A Systematic Review and Individual Patient Data Meta-Analysis. *J Clin Periodontol* 2013; 40: 607-615. doi: 10.1111/jcpe.12106

Chambrone L, Preshaw P.M., Ferreira J.D., Rodrigues F.A., Cassoni A, Shibli J.A. Effects of Tobacco Smoking on the Survival Rate of Dental Implants Placed in Areas of Maxillary Sinus Floor Augmentation: A Systmatic Review. *Clin Oral Impl Res* 2014; 25:408-416.

Chávarry N. G. M. (2009) The relationship between diabetes mellitus and destructive periodontal disease: a meta-analysis. *Oral health & preventive dentistry* 7, 107-27.

Chikazu D., Katagiri M., Ogasawara T., Ogata N., Shimoaka T., Takato T., Nakamura K. & Kawaguchi H. (2001) Regulation of osteoclast differentiation by fibroblast growth factor 2: stimulation of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor expression in osteoblasts and inhibition of macrophage colony-stimulating factor function in osteoclast precursors. *Journal of bone and mineral research*

: the official journal of the American Society for Bone and Mineral Research 16, 2074-2081. doi: 10.1359/jbmr.2001.16.11.2074

Coffin J. D., Florkiewicz R. Z., Neumann J., Mort-Hopkins T., Dorn G. W., 2nd, Lightfoot P., German R., Howles P. N., Kier A. & O'Toole B. A. (1995) Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Molecular biology of the cell* 6, 1861-1873.

de Morais, Juliana Aparecida Najarro Dearo. (2009) Effect of diabetes mellitus and insulin therapy on bone density around osseointegrated dental implants: a digital subtraction radiography study in rats. *Clinical oral implants research* 20, 796-801.

De Oliveira N. F., Andia D. C., Planello A. C., Pasetto S., Marques M. R., Nociti F. H., Jr, Line S. R. & De Souza A. P. (2011) TLR2 and TLR4 gene promoter methylation status during chronic periodontitis. *Journal of clinical periodontology* 38, 975-983. doi: 10.1111/j.1600-051X.2011.01765.x; 10.1111/j.1600-051X.2011.01765.x

Desta T. (2010) Altered fibroblast proliferation and apoptosis in diabetic gingival wounds. *Journal of dental research* 89, 609-14.

Devlin H. (1996) Healing of tooth extraction sockets in experimental diabetes mellitus. *Journal of oral and maxillofacial surgery* 54, 1087-91.

Divine K. K., Pulling L. C., Marron-Terada P. G., Liechty K. C., Kang T., Schwartz A. G., Bocklage T. J., Coons T. A., Gilliland F. D. & Belinsky S. A. (2005) Multiplicity of abnormal promoter methylation in lung adenocarcinomas from smokers and never smokers. *International journal of cancer. Journal international du cancer* 114, 400-405. doi: 10.1002/ijc.20761

Emrich L. J. (1991) Periodontal disease in non-insulin-dependent diabetes mellitus. *Journal of periodontology* (1970) 62, 123-31.

Fei Y., Xiao L., Doetschman T., Coffin D. J. & Hurley M. M. (2011) Fibroblast growth factor 2 stimulation of osteoblast differentiation and bone formation is mediated by modulation of the Wnt signaling pathway. *The Journal of biological chemistry* 286, 40575-40583. doi: 10.1074/jbc.M111.274910

Fei Y, Gronowicz G, Hurley M. Fibroblast Growth Factor-2, Bone Homeostasis and Fracture Repair. *Current Pharmaceutical Design*, 2013, 19, 3354-3363.

Fiorini T, Musskopf M. L., Opperman R. V., Susin C. (2014) Is There a Positive Effect of Smoking Cessation on Periodontal Health? A Systematic Review. *J Periodontology* 2014; 85: 83-91.

Furuya H, Tabata Y, Kaneko K. *Tissue Eng. A* 20 (2014) 1531-1541.

- Giannopoulou C., Geinoz A. & Cimasoni G. (1999) Effects of nicotine on periodontal ligament fibroblasts in vitro. *Journal of clinical periodontology* 26, 49-55.
- Glickman I. (1967) Postsurgical periodontal healing in alloxan diabetes. *Journal of periodontology* (1970) 38, 93-9.
- Gomez R. S., Dutra W. O. & Moreira P. R. (2009) Epigenetics and periodontal disease: future perspectives. *Inflammation research : official journal of the European Histamine Research Society ...[et al.]* 58, 625-629. doi: 10.1007/s00011-009-0041-7
- Gooch H. L. (2000) Alterations of cartilage and collagen expression during fracture healing in experimental diabetes. *Connective tissue research* 41, 81-91.
- Gospodarowicz D. (1974) Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature* 249, 123-127.
- Haffajee A. D. & Socransky S. S. (2001) Relationship of cigarette smoking to attachment level profiles. *Journal of clinical periodontology* 28, 283-295.
- Han W., Wang T., Reilly A. A., Keller S. M. & Spivack S. D. (2009) Gene promoter methylation assayed in exhaled breath, with differences in smokers and lung cancer patients. *Respiratory research* 10, 86. doi: 10.1186/1465-9921-10-86
- Hanada K., Dennis J. E. & Caplan A. I. (1997) Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 12, 1606-1614. doi: 10.1359/jbmr.1997.12.10.1606
- Heitz-Mayfield L. JA, Huynh-Ba G. (2009) History of Treated Periodontitis and Smoking as Risks for Implant Therapy. *Journal of Oral and Maxillofacial Implants* 24 (suppl): 39-68.
- Henemyre C. L., Scales D. K., Hokett S. D., Cuenin M. F., Peacock M. E., Parker M. H., Brewer P. D. & Chuang A. H. (2003) Nicotine stimulates osteoclast resorption in a porcine marrow cell model. *Journal of periodontology* 74, 1440-1446. doi: 10.1902/jop.2003.74.10.1440
- Horswill M. A., Narayan M., Warejcka D. J., Cirillo L. A. & Twining S. S. (2008) Epigenetic silencing of maspin expression occurs early in the conversion of keratocytes to fibroblasts. *Experimental eye research* 86, 586-600. doi: 10.1016/j.exer.2008.01.003
- Javed F. (2009) Impact of diabetes mellitus and glycemic control on the osseointegration of dental implants: a systematic literature review. *Journal of periodontology* 80, 1719-30.

Johnson G. K. & Guthmiller J. M. (2007) The impact of cigarette smoking on periodontal disease and treatment. *Periodontology* 2000 44, 178-194. doi: 10.1111/j.1600-0757.2007.00212.x

Kaigler D., Cirelli J. A. & Giannobile W. V. (2006) Growth factor delivery for oral and periodontal tissue engineering. *Expert opinion on drug delivery* 3, 647-662. doi: 10.1517/17425247.3.5.647

Kawaguchi H, Kurokawa T, Hanada K, Hiyama Y, Tamura M, Ogata E, Matsumoto T. *Endocrinology* 135 (1994) 774-781.

Kawaguchi H., Chikazu D., Nakamura K., Kumegawa M. & Hakeda Y. (2000) Direct and indirect actions of fibroblast growth factor 2 on osteoclastic bone resorption in cultures. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 15, 466-473. doi: 10.1359/jbmr.2000.15.3.466

Kawaguchi H., Pilbeam C. C., Gronowicz G., Abreu C., Fletcher B. S., Herschman H. R., Raisz L. G. & Hurley M. M. (1995) Transcriptional induction of prostaglandin G/H synthase-2 by basic fibroblast growth factor. *The Journal of clinical investigation* 96, 923-930. doi: 10.1172/JCI118140

Khader Y. S. (2006) Periodontal status of diabetics compared with nondiabetics: a meta-analysis. *Journal of diabetes and its complications* 20, 59-68.

Kitamura M., Akamatsu M., Machigashira M., Hara Y., Sakagami R., Hirofuji T., Hamachi T., Maeda K., Yokota M., Kido J., Nagata T., Kurihara H., Takashiba S., Shibutani T., Fukuda M., Noguchi T., Yamazaki K., Yoshie H., Ioroi K., Arai T., Nakagawa T., Ito K., Oda S., Izumi Y., Ogata Y., Yamada S., Shimauchi H., Kunimatsu K., Kawanami M., Fujii T., Furuichi Y., Furuuchi T., Sasano T., Imai E., Omae M., Yamada S., Watanuki M. & Murakami S. (2011) FGF-2 stimulates periodontal regeneration: results of a multi-center randomized clinical trial. *Journal of dental research* 90, 35-40. doi: 10.1177/0022034510384616

Kitamura M., Nakashima K., Kowashi Y., Fujii T., Shimauchi H., Sasano T., Furuuchi T., Fukuda M., Noguchi T., Shibutani T., Iwayama Y., Takashiba S., Kurihara H., Ninomiya M., Kido J., Nagata T., Hamachi T., Maeda K., Hara Y., Izumi Y., Hirofuji T., Imai E., Omae M., Watanuki M. & Murakami S. (2008) Periodontal tissue regeneration using fibroblast growth factor-2: randomized controlled phase II clinical trial. *PloS one* 3, e2611. doi: 10.1371/journal.pone.0002611

Krishnan V., Bryant H. U. & Macdougald O. A. (2006) Regulation of bone mass by Wnt signaling. *The Journal of clinical investigation* 116, 1202-1209. doi: 10.1172/JCI28551

Kumar A, Jaffin R. A., Berman C (2002). The Effect of Smoking on Achieving Osseointegration of Surface-Modified Implants: A Clinical Report. *Int J Oral Maxillofac Implants* 2002; 17: 816-819.

Kuroda A., Rauch T. A., Todorov I., Ku H. T., Al-Abdullah I. H., Kandeel F., Mullen Y., Pfeifer G. P. & Ferreri K. (2009) Insulin gene expression is regulated by DNA methylation. *PloS one* 4, e6953. doi: 10.1371/journal.pone.0006953

Labriola A., Needleman I. & Moles D. R. (2005) Systematic review of the effect of smoking on nonsurgical periodontal therapy. *Periodontology* 2000 37, 124-137. doi: 10.1111/j.1600-0757.2004.03793.x

Lang NP, Salvi GE, Huynh-Ba G, Ivanovski S, Donos N, Bosshardt DD. Early osseointegration to hydrophilic and hydrophobic implant surfaces in hamms. *CLin Oral IMplants Res* 2011; 22: 349-356.

Lee J., Stavropoulos A., Susin C. & Wikesjo U. M. (2010) Periodontal regeneration: focus on growth and differentiation factors. *Dental clinics of North America* 54, 93-111. doi: 10.1016/j.cden.2009.09.001

Lin R. K., Hsieh Y. S., Lin P., Hsu H. S., Chen C. Y., Tang Y. A., Lee C. F. & Wang Y. C. (2010) The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation and tumor suppressor gene hypermethylation in mice and lung cancer patients. *The Journal of clinical investigation* 120, 521-532. doi: 10.1172/JCI40706; 10.1172/JCI40706

Ling C. & Groop L. (2009) Epigenetics: a molecular link between environmental factors and type 2 diabetes. *Diabetes* 58, 2718-2725. doi: 10.2337/db09-1003

Ling C., Del Guerra S., Lupi R., Ronn T., Granhall C., Luthman H., Masiello P., Marchetti P., Groop L. & Del Prato S. (2008) Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. *Diabetologia* 51, 615-622. doi: 10.1007/s00125-007-0916-5

Liu Y., Gao W., Siegfried J. M., Weissfeld J. L., Luketich J. D. & Keohavong P. (2007) Promoter methylation of RASSF1A and DAPK and mutations of K-ras, p53, and EGFR in lung tumors from smokers and never-smokers. *BMC cancer* 7, 74. doi: 10.1186/1471-2407-7-74

Lou X, Wang H, Xia S, Skog S, Sun J (2014). Effects of Resveratrol on the Expression and DNA Methylation of Cytokine Genes in Diabetic Rat Aortas. *Arch Immunol Ther Exp* 2014; 62: 329-340.

Lu H. (2003) Diabetes interferes with the bone formation by affecting the expression of transcription factors that regulate osteoblast differentiation. *Endocrinology (Philadelphia)* 144, 346-52.

Lu J., Jiang W., Yang J. H., Chang P. Y., Walterscheid J. P., Chen H. H., Marcelli M., Tang D., Lee Y. T., Liao W. S., Yang C. Y. & Chen C. H. (2008) Electronegative LDL

impairs vascular endothelial cell integrity in diabetes by disrupting fibroblast growth factor 2 (FGF2) autoregulation. *Diabetes* 57, 158-166. doi: 10.2337/db07-1287

Machuca G., Rosales I., Lacalle J. R., Machuca C. & Bullon P. (2000) Effect of cigarette smoking on periodontal status of healthy young adults. *Journal of periodontology* 71, 73-78. doi: 10.1902/jop.2000.71.1.73

Maegawa N., Kawamura K., Hirose M., Yajima H., Takakura Y. & Ohgushi H. (2007) Enhancement of osteoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2). *Journal of tissue engineering and regenerative medicine* 1, 306-313. doi: 10.1002/term.41

Manouchehr-Pour M. (1981) Comparison of neutrophil chemotactic response in diabetic patients with mild and severe periodontal disease. *Journal of periodontology* (1970) 52, 410-5.

Marie P. J. (2003) Fibroblast growth factor signaling controlling osteoblast differentiation. *Gene* 316, 23-32.

McCarthy A. D. (2001) Non-enzymatic glycosylation of a type I collagen matrix: effects on osteoblastic development and oxidative stress. *BMC cell biology* 2, 16.

McMullen J. A. (1981) Neutrophil chemotaxis in individuals with advanced periodontal disease and a genetic predisposition to diabetes mellitus. *Journal of periodontology* (1970) 52, 167-73.

Mealey B. L. (2006) Diabetes mellitus and periodontal diseases. *Journal of periodontology* 2006; 77, 1289-1303.

Minamide A., Yoshida M., Kawakami M., Okada M., Enyo Y., Hashizume H. & Boden S. D. (2007) The effects of bone morphogenetic protein and basic fibroblast growth factor on cultured mesenchymal stem cells for spine fusion. *Spine* 32, 1067-1071. doi: 10.1097/01.brs.0000261626.32999.8a

Monnier V. M. (1996) The mechanism of collagen cross-linking in diabetes: a puzzle nearing resolution. *Diabetes* (New York, N.Y.) 45 Suppl 3, S67-72.

Montero A., Okada Y., Tomita M., Ito M., Tsurukami H., Nakamura T., Doetschman T., Coffin J. D. & Hurley M. M. (2000) Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. *The Journal of clinical investigation* 105, 1085-1093. doi: 10.1172/JCI8641

Morita I. (2012) Relationship between Periodontal Status and Levels of Glycated Hemoglobin. *Journal of dental research* 91, 161-6.

Murakami S. (2011) Periodontal tissue regeneration by signaling molecule(s): what role does basic fibroblast growth factor (FGF-2) have in periodontal therapy? *Periodontology* 2000 56, 188-208. doi: 10.1111/j.1600-0757.2010.00365.x; 10.1111/j.1600-0757.2010.00365.x

Murakami S., Takayama S., Kitamura M., Shimabukuro Y., Yanagi K., Ikezawa K., Saho T., Nozaki T. & Okada H. (2003) Recombinant human basic fibroblast growth factor (bFGF) stimulates periodontal regeneration in class II furcation defects created in beagle dogs. *Journal of periodontal research* 38, 97-103.

Murakami S., Takayama S., Ikezawa K., Shimabukuro Y., Kitamura M., Nozaki T., Terashima A., Asano T. & Okada H. (1999) Regeneration of periodontal tissues by basic fibroblast growth factor. *Journal of periodontal research* 34, 425-430.

Murphy S. K., Adigun A., Huang Z., Overcash F., Wang F., Jirtle R. L., Schildkraut J. M., Murtha A. P., Iversen E. S. & Hoyo C. (2011) Gender-specific methylation differences in relation to prenatal exposure to cigarette smoke. *Gene* doi: 10.1016/j.gene.2011.11.062

Nagayasu-Tanaka T, Nozaki T, Miki K, Sawada K, Kitamura M, Murakami S. FGF-2 promotes initial osseointegration and enhances stability of implants with low primary stability. *Clin Oral Impl Res* 00, 2016, 1-7.

Naguib G. (2004) Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation. *Journal of investigative dermatology* 123, 87-92.

Natto S., Baljoon M. & Bergstrom J. (2005) Tobacco smoking and periodontal bone height in a Saudi Arabian population. *Journal of clinical periodontology* 32, 1000-1006. doi: 10.1111/j.1600-051X.2005.00813.x

Nelson R. G. (1990) Periodontal disease and NIDDM in Pima Indians. *Diabetes care* 13, 836-40.

Nishimura F. (1996) Glucose-mediated alteration of cellular function in human periodontal ligament cells. *Journal of dental research* 75, 1664-71.

Ohi T., Uehara Y., Takatsu M., Watanabe M. & Ono T. (2006) Hypermethylation of CpGs in the promoter of the COL1A1 gene in the aged periodontal ligament. *Journal of dental research* 85, 245-250.

Oliveira N. F., Damm G. R., Andia D. C., Salmon C., Nociti F. H., Jr, Line S. R. & de Souza A. P. (2009) DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. *Journal of clinical periodontology* 36, 719-725. doi: 10.1111/j.1600-051X.2009.01446.x

- Pabst M. J., Pabst K. M., Collier J. A., Coleman T. C., Lemons-Prince M. L., Godat M. S., Waring M. B. & Babu J. P. (1995) Inhibition of neutrophil and monocyte defensive functions by nicotine. *Journal of periodontology* 66, 1047-1055.
- Palmer R. M., Wilson R. F., Hasan A. S. & Scott D. A. (2005) Mechanisms of action of environmental factors--tobacco smoking. *Journal of clinical periodontology* 32 Suppl 6, 180-195. doi: 10.1111/j.1600-051X.2005.00786.x
- Park J. B. (2011) Effects of fibroblast growth factor 2 on osteoblastic proliferation and differentiation by regulating bone morphogenetic protein receptor expression. *The Journal of craniofacial surgery* 22, 1880-1882. doi: 10.1097/SCS.0b013e31822e8434
- Pauwels N. S., Bracke K. R., Maes T., Van Pottelberge G. R., Garlanda C., Mantovani A., Joos G. F. & Brusselle G. G. (2010) Cigarette smoke induces PTX3 expression in pulmonary veins of mice in an IL-1 dependent manner. *Respiratory research* 11, 134. doi: 10.1186/1465-9921-11-134
- Pinto J. R., Bosco A. F., Okamoto T., Guerra J. B. & Piza I. G. (2002) Effects of nicotine on the healing of extraction sockets in rats. A histological study. *Brazilian dental journal* 13, 3-9.
- Reddy M. A. & Natarajan R. (2011) Epigenetics in diabetic kidney disease. *Journal of the American Society of Nephrology : JASN* 22, 2182-2185. doi: 10.1681/ASN.2011060629
- Rosa G. M., Lucas G. Q. & Lucas O. N. (2008) Cigarette smoking and alveolar bone in young adults: a study using digitized radiographs. *Journal of periodontology* 79, 232-244. doi: 10.1902/jop.2008.060522
- Rossa C., Jr, Marcantonio E., Jr, Cirelli J. A., Marcantonio R. A., Spolidorio L. C. & Fogo J. C. (2000) Regeneration of Class III furcation defects with basic fibroblast growth factor (b-FGF) associated with GTR. A descriptive and histometric study in dogs. *Journal of periodontology* 71, 775-784. doi: 10.1902/jop.2000.71.5.775
- Santana R. B. (2003) A role for advanced glycation end products in diminished bone healing in type 1 diabetes. *Diabetes (New York, N.Y.)* 52, 1502-10.
- Schmidt A. M. (1999) Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. *Circulation research* 84, 489-97.
- Schmidt A. M. (1996) RAGE: a novel cellular receptor for advanced glycation end products. *Diabetes (New York, N.Y.)* 45 Suppl 3, S77-80.
- SILNESS J. & LOE H. (1964) Periodontal Disease in Pregnancy. Ii. Correlation between Oral Hygiene and Periodontal Condition. *Acta Odontologica Scandinavica* 22, 121-135.

Simmons H. A. & Raisz L. G. (1991) Effects of acid and basic fibroblast growth factor and heparin on resorption of cultured fetal rat long bones. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 6, 1301-1305. doi: 10.1002/jbmr.5650061206

Singh K, Agrawal N.K., Gupta S.K., Mohan G, Chaturvedi S, Singh K. (2015). Genetic and Epigenetic Alterations in Toll like receptor 2 and wound healing impairment in Type 2 diabetes patients. *Journal of Diabetes and Its Complications* 2015; 29:222-229

Soria J. C., Rodriguez M., Liu D. D., Lee J. J., Hong W. K. & Mao L. (2002) Aberrant promoter methylation of multiple genes in bronchial brush samples from former cigarette smokers. *Cancer research* 62, 351-355.

Sorsa T. (1992) Cellular source and tetracycline-inhibition of gingival crevicular fluid collagenase of patients with labile diabetes mellitus. *Journal of clinical periodontology* 19, 146-9.

Southerland J. H., Taylor G. W., Moss K., Beck J. D. & Offenbacher S. (2006) Commonality in chronic inflammatory diseases: periodontitis, diabetes, and coronary artery disease. *Periodontology* 2000 40, 130-143. doi: 10.1111/j.1600-0757.2005.00138.x

Southerland J. H. (2006) Commonality in chronic inflammatory diseases: periodontitis, diabetes, and coronary artery disease. *Periodontology* 2000 40, 130-43.

Strietzel F.P, Reichart P.A., Kale A., Kulkarni M., Wegner B., Kuchler I (2007) Smoking interferes with the prognosis of dental implant treatment: a systematic review and meta-analysis. *J Clin Periodontol* 2007; 34: 523-544.

Takayama S., Murakami S., Shimabukuro Y., Kitamura M. & Okada H. (2001) Periodontal regeneration by FGF-2 (bFGF) in primate models. *Journal of dental research* 80, 2075-2079.

Tanaka T, Kitasato S, Chazono M, Kumagae Y, Lida T, Mitsuhashi M, Kakuta A, Marumo K. *Open Biomed Eng. J.* 6 (2012) 98-103.

Taylor G. W. (2001) Bidirectional interrelationships between diabetes and periodontal diseases: an epidemiologic perspective. *Annals of periodontology* 6, 99-112.

Taylor G. W. (1998) Non-insulin dependent diabetes mellitus and alveolar bone loss progression over 2 years. *Journal of periodontology* (1970) 69, 76-83.

Tekpli X., Zienolddiny S., Skaug V., Stangeland L., Haugen A. & Mollerup S. (2011) DNA methylation of the CYP1A1 enhancer is associated with smoking-induced genetic alterations in human lung. *International journal of cancer. Journal international du cancer* doi: 10.1002/ijc.27421; 10.1002/ijc.27421

Theiss S. M., Boden S. D., Hair G., Titus L., Morone M. A. & Ugbo J. (2000) The effect of nicotine on gene expression during spine fusion. *Spine* 25, 2588-2594.

Tipton D. A. & Dabbous M. K. (1995) Effects of nicotine on proliferation and extracellular matrix production of human gingival fibroblasts in vitro. *Journal of periodontology* 66, 1056-1064.

Tomar S. L. & Asma S. (2000) Smoking-attributable periodontitis in the United States: findings from NHANES III. National Health and Nutrition Examination Survey. *Journal of periodontology* 71, 743-751. doi: 10.1902/jop.2000.71.5.743

Tymkiw K., Thunell D., Johnson G., Joly S., Burnell K., Cavanaugh J., Brogden K. & Guthmiller J. (2011) Influence of smoking on gingival crevicular fluid cytokines in severe chronic periodontitis. *J Clin Periodontol* 38, 219-228.

Wautier J. L. (1998) Diabetes, advanced glycation endproducts and vascular disease. *Vascular medicine (London, England)* 3, 131-7.

Werner S., Breiden M., Hubner G., Greenhalgh D. G. & Longaker M. T. (1994) Induction of keratinocyte growth factor expression is reduced and delayed during wound healing in the genetically diabetic mouse. *The Journal of investigative dermatology* 103, 469-473.

Wilson A. G. (2008) Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases. *Journal of periodontology* 79, 1514-1519. doi: 10.1902/jop.2008.080172

Winer N. & Sowers J. R. (2004) Epidemiology of Diabetes. *J clin pharmacol* 44, 397.

Zhang B., Zhu W., Yang P., Liu T., Jiang M., He Z. N., Zhang S. X., Chen W. Q. & Chen W. (2011) Cigarette Smoking and p16 Gene Promoter Hypermethylation in Non-Small Cell Lung Carcinoma Patients: A Meta-Analysis. *PloS one* 6, e28882. doi: 10.1371/journal.pone.0028882

Zhang S., Barros S. P., Niculescu M. D., Moretti A. J., Preisser J. S. & Offenbacher S. (2010) Alteration of PTGS2 promoter methylation in chronic periodontitis. *Journal of dental research* 89, 133-137. doi: 10.1177/0022034509356512

Zhao J., Goldberg J., Bremner J. D. & Vaccarino V. (2011) Global DNA Methylation Is Associated With Insulin Resistance: A Monozygotic Twin Study. *Diabetes* doi: 10.2337/db11-1048